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MOLECULAR AND MECHANICAL CONTROL OF PLANT SECONDARY DEVELOPMENT

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Doctoral Thesis

Molecular and mechanical control of plant secondary development

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1. List of Original Publications

I. **Alonso-Serra J**, Shi X[1], Peaucelle A[1], Rastas P[1], Bourdon M[1], Immanen J, Takahashi J, Koivula H, Eswaran G, Muranen S, Help-Rinta-Rahko H, Smolander O-P, Su C, Safronov O, Gerber L, Salojarvi J, Hagqvist R, Mahonen A-P, Helariutta Y & Nieminen K. *ELIMÄKI* locus is required for vertical proprioceptive response in birch trees. *Current Biology* (2020).

II. **Alonso-Serra J[1]**, Safronov O[1], Lim K-J[1], Fraser-Miller SJ, Blokhina OB, Campilho A, Chong S-L, Fagerstedt K, Haavikko R, Helariutta Y, Immanen J, Kangasjärvi J, Kauppila TJ, Lehtonen M, Ragni L, Rajaraman S, Räsänen R-M, Safdari P, Tenkanen M, Yli-Kauhaluoma JT & Salojärvi J. Tissue-specific study across the stem reveals the chemistry and transcriptome dynamics of birch bark. *New Phytologist* (2019).

III. Jing Zhang, Gugan Eswaran[1], **J Alonso-Serra[1]**, Melis Kucukoglu, Jiale Xiang, Weibing Yang, Annakaisa Elo, Kaisa Nieminen, Teddy Damén, Je-Gun Joung, Jae-Young Yun, Jung-Hun Lee, Laura Ragni, Pierre B. de Reuille, Sebastian E. Ahnert, Ji-Young Lee, Ari Pekka Mähönen and Ykä Helariutta. Transcriptional regulatory framework for vascular cambium development in *Arabidopsis* roots. *Nature Plants* (2019).

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2. Author's contributions

I) Y.H, K.N and **J.A.S.**, designed the research; **J.A.S**, X.S, A.P, P.R, M.B, J.I, J.T, H.K, G.E, S.M, H.H, O-P.S, C.S, R.H, A-P.M and K.N performed the research; **J.A.S**, X.S, A.P, P.R, M.B, J.I, J.T, H.K, G.E, O-P.S, L.G, O.S, J.S and K.N analyzed the data; and **J.A.S**, Y.H and K.N wrote the paper.

II) Y.H., J.K., M.T, J.Y.K., T.H.T., K.F., **J.A.S.**, O.S., J.I., T.J.K., R.M.R., C.J.S., K.N., J.S. designed the research; K.J.L., **J.A.S** performed the RNA extractions. **J.A.S**, K.N., J.I. did the sampling for GCMS and FTIR and produced all anatomical pictures. O.S., J.S. carried out the analysis of transcriptomic data, and O.S., **J.A.S**, P.S., O.B.B., L.R., A.C., S.R., J.S. participated in the interpretation and identification of candidate genes. **J.A.S**, O.S., K.N., C.J.S., J.S. wrote the manuscript with input from S.J.M., KJL, O.B.B., Y.H.

III) **J.A.S.**, G.E. and J.Z. carried out the experiments combining mutant and overexpression lines. For phenotypic examination, J.Z., G.E. and **J.A.S** conducted quantification and statistical analyses. **J.A.S** ran LithoGraphX analysis with the input from G.E. **J.A.S** contributed with writing the manuscript.

3. Abbreviations

SAM	shoot apical meristem
RAM	root apical meristem
TFs	transcription factors
WOX	WUSCHEL HOMEOBOX RELATED
KNAT	KNOTTED-LIKE FROM ARABIDOPSIS THALIANA
MP	MONOPTEROS
ARF	AUXIN RESPONSE FACTOR
TMO	TARGET OF MONOPTEROS
LHW	LONESOMEHIGHWAY
LOG	LONELY GUY
AHP	ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN
PEAR	PHLOEM EARLY DOF
CLV	CLAVATA
CLE	CLAVATA EMBRYO SURROUNDING REGION-RELATED
PXY	PHLOEM INTERCALATED WITH XYLEM
PtSHR2B	*Populus trichocarpa* SHORT ROOT 2B
FER	FERRONIA
THE	THESEUS
CUC	CUP-SHAPED COTYLEDON
STM	SHOOTMERISTEMLESS
PtaZFP	*Populus tremula* × *Populus alba* ZINC FINGER PROTEIN
MYB	MYB DOMAIN PROTEIN
LBD	LOB DOMAIN-CONTAINING PROTEIN
ERF	ETHYLENE RESPONSE FACTOR
PTL	PETALLOSS

4. Abstract

Plant development takes place through continuous changes in the size and shape of organs. Along the organs' morphogenic gradients, cells derived from the undifferentiated meristematic stem cells follow different regulatory pathways leading to a variety of developmental trajectories and cellular functions. In the developmental process called secondary growth, molecular factors and physical forces interact in the radial growth of stems and roots to produce their cylindrical shape. Cambium, the largest connected meristem in plants, is responsible for secondary growth. It produces vascular tissues with two essential functions: the transport of water, nutrients and photoassimilates, and the physical support of the plant. Recent years have seen an increasing number of studies focused on the regulation of cambial activity, primarily because this meristem produces a great part of the Earth's woody biomass, thereby fixing a large quantity of carbon. The aim of this thesis is to explore aspects of radial growth which have thus far remained largely uncharacterized: the contribution of bark tissue, the role of mechanical forces, and the genetic robustness of cambial development.

Tiivistelmä

Kasvin kehitys tapahtuu sen eri osien koon ja muodon jatkuvien muutosten kautta. Nämä jatkuvat muutokset näkyvät osien rakenteellisissa vyöhykkeissä. Kasvi kasvaa sen kasvusolukkojen erilaistumattomien kantasolujen tuottamien uusien solujen avulla. Nämä solut altistuvat erilaisille säätelyreiteille, jotka johtavat niiden erilaistumiseen omille kehityskuluilleen ja toiminnoilleen.

Paksuuskasvussa molekulaariset tekijät ja fysikaaliset voimat toimivat yhdessä ja tuottavat varren ja juurien lieriömäisen muodon. Jälsi, joka on kasvin suurin yhtenäinen kasvusolukko, vastaa sen eri osien paksuuskasvusta. Tämä

kasvusolukko tuottaa johtosolukoita, joilla on kaksi tärkeää tehtävää: veden, ravinteiden ja yhteytystuotteiden kuljetus sekä kasvin rakenteellinen tuki.

Viime vuosina jällen toiminnan säätelyä selvittävien tutkimusten määrä on kasvanut: lisääntynyt kiinnostus heijastaa tämän kasvusolukon suurta merkitystä maapallon puubiomassan tuottamiselle ja sitä myötä hiilen sidonnalle. Väitöskirjani tavoitteena on ollut tutkia kasvien paksuuskasvun nykyisellään vähän tunnettuja puolia: kuorisolukoiden, mekaanisten voimien ja geneettisen säätelyverkoston osuutta jällen toiminnassa.

5. Introduction

On the problem of plant blindness

If we imagine a landscape and dissect its composition, whether a small urban garden or a vast forest, we will undoubtedly acknowledge that its value originates from two sources: the subjective experience of the observer and the diversity of the world. The diversity within the plant kingdom shapes and strengthens ecosystems by building interdependent networks with fungi, bacteria and a broad range of pollinators and seed-dispersing animals. Even in the most hostile environments on Earth, the evolution of plant species has selected a remarkable variety of adaptive characteristics and strategies that succeed in passing genes on to the next generation. Today, this ecological diversity across kingdoms faces an unprecedented risk of extinction, revealing the consequences of a broken ecological equilibrium across the globe (Media Release: Nature's Dangerous Decline 'Unprecedented'; Species Extinction Rates 'Accelerating' | IPBES; Humphreys *et al*, 2019). Therefore, in addition to ongoing conservation efforts, we are faced with the challenging task of recognizing value in these ecosystems beyond their evident function, productivity and beauty. For example, many

indigenous groups consider trees to be members of their community and part of their identity (West, 2005).

Even though plants are essential for human life and represent the largest living biomass on Earth (Bar-On *et al*, 2018), our anthropocentric perspective frequently alienates *us* from *nature*. The perception of plants as sessile organisms, with just a few visible movements, seemingly enhances our separation and positions plants as merely decorative or productive objects in our societies.

However, a forest does not emerge overnight. The final size, shape and colors of such an ecosystem are the expression of a genetic bottle containing more than 400 million years of evolution since the colonization of land by plants. The diverse morphologies of plant organs and species are also shaped during development by the local environment each individual encounters. If we replace our mental snapshot with a time-lapse video showing developmental changes, we might recognize that, though fixed in place, plants are far from static. By constantly adjusting their shape and size, they add robustness to their adaptation and augment it with enormous developmental plasticity.

In this framework, the study of plant development offers a powerful approach to remove the veil cast over our eyes by the different tempos of plants and people. Through our studies, we can better understand how distinct morphologies arise from similar-looking embryos. Therefore, this field not only offers a window into the hidden motions and gradual changes in the life of plants but can also alter the perspective of the observer by replacing the snapshot view of our senses with a more complex and dynamic understanding.

5.1 Origins of the plant vascular system

Over time, the plant vascular system has evolved to support and feed large quantities of green biomass. Plants, and perhaps every living organism on Earth,

began their evolutionary path in the oceans. The transition from an aquatic environment to a terrestrial habitat occurred nearly 500 million years ago in the Cambrian period (Morris *et al*, 2018). This process increased the O_2 concentration in the atmosphere and reshaped the Earth's landscape. The new habitat, where water was supplied from the soil and carbon derived from the air, selected a novel diversity of life-forms with different functional requirements for their tissues. In fact, aerial and terrestrial organs were nutritionally independent in early multicellular plants, which may have favored advances in cellular communication (Lucas *et al*, 2013).

Fossil records suggest that pre-tracheophyte (non-vascular) land plants already had water-conducting cells. Among the earliest differentiated conductive cells to have evolved are hydroids, dead cells with unevenly thickened cell walls that permit the transport of water. Hydroids later evolved into the conductive tissue known as xylem. Another early cell type, named leptoids, originated as food-conducting cells. Leptoid were elongated cells with cytoplasmic polarity, partial degradation of the nucleus, and a high density of plasmodesmata (inter-cellular channels). Leptoids evolved into phloem tissues, more specifically into sieve elements, which are essential for the transport of photosynthates in vascular plants. Today, hydroids and leptoids are still present in some non-vascular plants, such as mosses; thus, the study of these organisms can offer an opportunity to explore the origin and evolution of vascular tissues. However, it is important to bear in mind that contemporary "simpler" organisms never stopped evolving, so they do not represent a static genetic snapshot of the past (Delaux *et al*, 2019).

The early evolution of these cell types indicates that there was positive selective pressure towards a connected system that would facilitate long-distance transport and vertical growth. The increasing distance between aerial and terrestrial organs inherently posed two new physical challenges, hydraulic conductivity and mechanical support, both of which were tackled in part by secondary growth.

The capacity to undergo secondary growth, to thicken organs by producing cylindrically connected rings of xylem inwards, has appeared multiple times in land plants (Spicer & Groover, 2010), representing a case of convergent evolution towards a support-based architecture. Even though it is thought that the cambium of all seeds plants is derived from a common ancestor, multiple adaptations of internal cambial organization can be found, from the classical cylinder observed in most trees to the non-continuous or compound rings observed in lianas, which results from non-uniform cambial growth (Tomescu & Groover, 2019). The cambial organization of plants such as lianas is thought to provide different mechanical properties tuned to their lifestyle in which a fast growing stem relies on a host tree for mechanical support (Groover, 2020).

Key events in the evolution of cell walls also facilitated the transport of water and nutrients. This specialized cover made of cellulose, hemicelluloses, lignins and proteins not only provides physical support but can also contain high hydraulic pressures. Complex cell walls with similar components can be found in both algae and land plants, and it is difficult to identify any land plant-specific cell wall polymer (Popper *et al*, 2011). For example, lignin was thought to be specific to terrestrial life habits, but traces of it have been identified in red algae (Martone *et al*, 2009). Therefore, it was likely the diversification and differentiation of cell wall types and matrix architectures rather than the evolution of new polymers that served as a source of new cellular functions and thus new life habits.

An example of this functional evolution can be found in woody cells. In gymnosperms such as conifers, xylem tissues consist almost purely of tracheids that simultaneously provide water transport capacity and support. In angiosperms, these functions are instead separated into two very different cell types: fibers and vessels, allowing the vessels to develop much greater water transport efficiency (Sperry, 2003).

5.2 Plant secondary development

5.2.1 Ontogeny of radial growth

Longitudinal or apical growth in plants originates from a continuous source of cell files in the root and shoot apical meristems (RAM and SAM). These developmental mechanisms are already initiated during embryogenesis, when the apical meristematic niches are established (Aichinger *et al*, 2012; De Rybel *et al*, 2016). While stem cells are capable of producing all tissues types derived from a given meristem, mother cells are specified to produce a certain lineage. In Arabidopsis roots, the quiescent center and the provasculature of the root meristem develop from inner cells located in the lower tissue of the fertilized zygote. These cells proliferate through periclinal and tangential divisions throughout the early globular stage of the embryo (ten Hove *et al*, 2015) and establish a bisymmetric organization composed of a longitudinal xylem axis, with central metaxylem and a protoxylem cell at each end, flanked by protophloem poles. The separating tissue between these two vascular tissues, procambium, later gives rise to the vascular cambium, which drives secondary growth once it is established and activated.

Two main interacting pathways have been identified through studies of the molecular and hormonal networks that coordinate the establishment of the vascular cambium The first consists of a negative feedback loop between auxin and cytokinin (Bishopp *et al*, 2011). In this regulatory network, cytokinin signaling originating from the protophloem and procambial tissues promotes the relocalization of PIN proteins to restrict auxin to the metaxylem axis. In turn, auxin promotes the expression of *AHP6* (Mähönen *et al*, 2006), a cytokinin inhibitor which excludes cytokinin signaling from the protoxylem tissues. The second pathway, which has been described more recently, also involves hormonal crosstalk between auxin and cytokinin. Here, the auxin-dependent

expression of two transcription factors *TMO5* and *LHW*, is modulated by another transcription factor named *MP/ARF5*. Interestingly, *TMO5* and *LHW* regulate the transcription of genes involved in the activation of cytokinin, *LOG* 3 and *LOG4*, whose activity further enhances cytokinin signaling (Ohashi-Ito *et al*, 2014; De Rybel *et al*, 2013).

In both pathways, genetic analyses have shown that the tissue-specific expression of transcription factors combined with precisely regulated hormonal signaling is essential to modulate the earliest establishment of procambial cells and their initial periclinal divisions. A recent finding has deepened our understanding of the initiation of radial growth by revealing key transcription factors controlling procambium development, namely *PEAR1* and *PEAR2* (Miyashima *et al*, 2019). These mobile, cytokinin-inducible transcription factors promote the earliest cell divisions in the phloem precursor cells, thereby driving the initial formation of procambium cells.

While the mechanisms controlling procambial and cambial growth have begun to be elucidated, the cellular and molecular events that occur during the developmental transition from the former to the latter still remain largely uncharacterized. Furthermore, the SAM-derived development of procambial tissues in the shoot remains understudied, aside from anatomical analyses (Serrano-Mislata & Sablowski, 2018). These gaps in our knowledge are a consequence of the fact that these secondary growth events occur in a section of the stem or root that has so far been inaccessible to microscopy *in vivo*.

5.2.2 Development of the vascular cambium

The vascular cambium is a cylindrically shaped meristem whose activity leads to radial thickening of the roots and shoots. When active, this bifacial meristem produces phloem tissues outwards and xylem tissues inwards (Chiang & Greb, 2019). Xylem tissues are not only responsible for the transport of nutrients and

water upwards from the roots but also provide physical support to the stem (Figure 1). In contrast, phloem tissues transport photoassimilates synthesized in the green tissues down to the roots, thereby supplying carbon to the entire plant architecture (López-Salmerón *et al*, 2019).

Cambium consists of a series of mother cells that can range from one (e.g. in young *Arabidopsis* roots) to dozens (in adult trees), but interestingly, both *Arabidopsis* and trees seem to maintain only a single stem cell in the center of cambium; this single cell retains the full capacity to produce both xylem and phloem tissues (Smetana *et al*, 2019; Bossinger & Spokevicius, 2018; Shi *et al*, 2019). The remaining cells in the cambial zone seem to be either transit amplifying cells or mother cells in which the initial tissue specification for a cell type identity has already occurred (Fischer *et al*, 2019).

More is known about the molecular regulation of the bifacial growth of cambium than about the regulation of its three-dimensional contribution to plant stem thickening. The current understanding of the molecular networks in cambium highlights a centripetal signaling cascade in which peptides synthesized in the phloem, such as CLE41 and CLE44, are perceived in the cambium by a receptor named PXY, which then promotes cambial cell division and restricts differentiation (Figure 2) (Etchells & Turner, 2010; Fisher & Turner, 2007; Hirakawa *et al*, 2008). Downstream of this pathway are the transcription factors *WOX4* and *WOX14*, which act as cambial regulators (Etchells *et al*, 2013). Interestingly, this pathway is important for controlling not only the rate of cambial cell divisions but also their orientation, given that *pxy* mutants and CLE41 gain-of-function genotypes display strong patterning defects. These genotypes can drastically disrupt the radial symmetry of the vascular tissues in the stem or root, but none of them affects the cylindrical shape of the stem, suggesting that additional molecular or physical factors control stem geometry.

Another important factor defining the identity of cells involved in secondary growth is their radial hormonal and transcriptional profile. In the *Arabidopsis* root, an auxin response on the xylem side of the cambium defines the cambial

organizing center through MP and induces the specification of the neighboring stem cell (Brackmann *et al*, 2018; Smetana *et al*, 2019). Similarly, hormonal measurements using tangential cryosections in trees have shown that the highest auxin concentration is on the xylem side of the cambium, while the cytokinin concentration peaks in the phloem (Immanen *et al*, 2016; Tuominen *et al*, 1997).

To our current understanding, unlike in apical meristems, where the loss of a single transcription factor can strongly affect primary growth and/or meristem maintenance (Long *et al*, 1996; Laux *et al*, 1996; Helariutta *et al*, 2000; Di Laurenzio *et al*, 1996), cambial activity cannot be completely abolished by the loss of any single transcription factor. Instead, the strongest known loss-of-function phenotype for cambial activity has been observed in a quadruple mutant of cytokinin biosynthesis isopentenyltransferase (IPT) enzymes (Matsumoto-Kitano *et al*, 2008), suggesting that the transcriptional regulation of cambial activity is robust and redundant. In trees, both the addition (Immanen *et al*, 2016) or removal (Nieminen *et al*, 2008) of cytokinin can strongly affect cambial growth and wood formation.

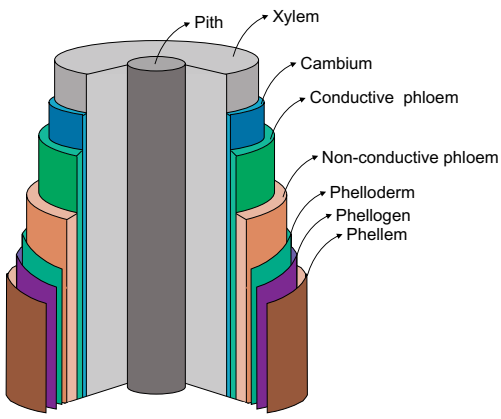

Figure 1: Three-dimensional organization of vascular tissues in the stem. Pith (center), xylem (wood), cambium, conductive phloem (photoassimilate transport), non-conductive phloem (phloem fibers and parenchymatic tissue), phelloderm, phellogen (cork cambium), phellem.

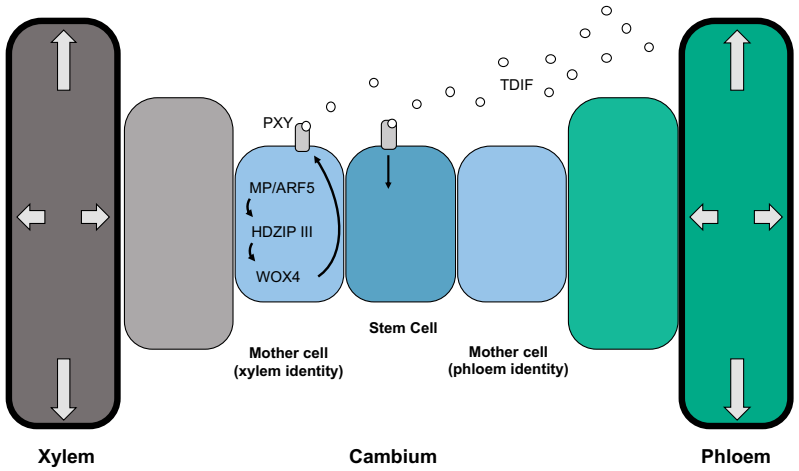

Figure 2: Simplified radial molecular regulation of the vascular cambium. PXY mediated signaling is activated by phloem secreted peptides (TDIF) to promote cell division in the cambium through WOX4. Auxin induced TFs (MP/ARF5) specify the xylem identity mother cells which correspond to the organizing center. The differentiation of xylem and phloem further enlarges cell size and connects these developing cells with the forming vascular system.

5.3 Tertiary meristems: The cork cambium

The tertiary meristem known as cork cambium is responsible for the development of the periderm, which in trees corresponds to the outermost layers of the bark tissues (bark proper comprises all the tissues outwards from the vascular cambium). The periderm consists of three anatomically and functionally distinct layers (Figure 1): the phellem (facing outwards), the phellogen or cork cambium (at the center) and the phelloderm (facing inwards) (Esau, 1977). The developmental dynamics of this meristem show remarkable variation among seasonal tree species. For example, in birch the cork cambium is established and remains at the outer bark layer, where it produces the hydrophobic phellem

tissues every summer. However, in cork oak (*Quercus suber*) the phellogen is re-specified every year in the inner bark, far from the surface, to initiate a new seasonal bark growth (Esau, 1977). This special characteristic makes cork oak bark suitable for harvesting for the cork industry. Since the phellem is directly exposed to environment, this tissue is highly specialized, typically having suberized cells rich in many secondary metabolites that provide an additional defense barrier against plant pathogens and herbivores. The phelloderm can have variable cell file numbers and functions. For example, in *Betula spp.* and *Fagus spp.*, this tissue is green and provides photosynthetic activity to the bark, which has been suggested to aid with the respiratory demands of the trunk (Wittmann & Pfanz, 2007; H. *et al*, 2002).

In the roots of *Arabidopsis*, the cork cambium originates from periclinal cell divisions of the pericycle cells in young roots, after which the production of phellem outwards and phelloderm inwards can be observed (Wunderling *et al*, 2018). Similar to the case in trees, the phellem cells in *Arabidopsis* are suberized and function as a protective barrier.

An interesting aspect of the periderm in both trees and *Arabidopsis* is its particular geometrical and biophysical characteristics. Like the epidermis, this tissue is under high tensile stress due to the radial growth of the inner tissues. In *Arabidopsis*, the radial growth of the stem can be strongly modulated by the capacity of the epidermis to divide (Savaldi-Goldstein *et al*, 2007), suggesting that this tissue may exert physical control over radial thickening. In the SAM, the epidermis can physically (due to its geometry) and molecularly modulate stem cell activity (Gruel *et al*, 2016); it remains to be determined whether the same is true of the stem periderm in trees.

Compared to vascular cambium, the molecular regulation of cork cambium development is less well understood (Campilho et al, 2019). To date, only one transcription factor has been functionally associated with the control of cork cambium activity, *PtSHR2B* in *Populus trichocarpa* (Miguel et al, 2016). Since its overexpression increases the proportion of bark tissues in the stem, this

transcription factor seems to positively regulate bark development.

5.4 Differences between *Arabidopsis* and trees

The regulation and developmental dynamics of cambial activity are remarkably well conserved between dioecious tree species and *Arabidopsis*, which has made *Arabidopsis* a highly relevant model species for secondary growth studies (Lens *et al*, 2012; Nieminen *et al*, 2004) and has significantly increased our understanding of wood formation.

However, weeds and trees have diverged multiple times throughout their evolution to develop different life habits and anatomical innovations. *Arabidopsis* plants can go through a complete life cycle in less than three months, whereas trees can live from hundreds to thousands of years. Due to the great difference in the time frame in which selective pressures can influence the production of the following generation, it is not surprising to find significant anatomical differences between trees and non-woody plants.

The largest section of the stem in *Arabidopsis* is occupied by the pith, a spongy tissue that consists of parenchyma cells. In angiosperm trees, this tissue is predominant only in early stages of stem development, after which xylem development largely dominates the cross-sectional area. Furthermore, cell types such as ray parenchyma and ray initials are missing in *Arabidopsis*. These cell types, which seem to be involved in the radial transport of metabolites (Blokhina *et al*, 2019), were an innovation of tree species. Another tree characteristic that is absent in *Arabidopsis* is the capacity to develop reaction wood under mechanical stress (Fournier *et al*, 2014). This developmental process involves the remodeling of cell walls to produce a cellulose-rich gel-like layer (in angiosperms) on the inner side of secondary cell walls (Lehringer *et al*, 2009; Gerttula *et al*, 2015).

Finally, a constitutive difference is the presence of seasonality in the cambial growth of trees. At the cellular level, the transition from an active to a dormant

state involves the reorganization of the cytoplasmic content, thickening of cell walls and vacuolar fragmentation (Lachaud *et al*, 1999).

In addition to the most popular current tree model species (*Populus spp.*), birch has emerged as a suitable species for basic and applied tree research. Silver birch (*Betula pendula*) has a relatively small diploid genome (440Mbp), is suitable for genetic transformation, can flower precociously, and since birches are monoecious, can be self-pollinated (Salojärvi *et al*, 2017; Longman & Wareing, 1959).

5.5 Physical modulators of plant morphogenesis

The connection between physics and growth has long been considered relevant for understanding plant and animal development (Thompson, 1917). However, the link between physical signals and molecular factors has only recently started to be explored.

Plant cell walls are typically under tensile stress resulting from the osmotic pressure of the intracellular environment (Mirabet *et al*, 2011). Similarly, the epidermis of plants is under tensile stress deriving from the expansion of the inner tissues (Verger *et al*, 2018). Both scenarios illustrate that cells and tissues are inherently pre-stressed components, and they must divide and differentiate in this context. At the same time, every cell and tissue grows at a slightly different rate and in a different direction and is differently affected by the larger mechanical environment (e.g. organ shape, growth direction, etc.). Interestingly, this heterogeneity, coupled with the feedback loop of physical signals, seems to add robustness to developmental processes, thereby creating reproducible shapes in nature (Hamant & Moulia, 2016; Hong *et al*, 2018).

Recent studies have found that the rate and direction of cell division in the SAM of *Arabidopsis* can be directed by physical forces and that microtubules play a central role in reading the direction of these signals (Louveaux *et al*, 2016; Uyttewaal *et al*, 2012; Hamant *et al*, 2008). Similarly, cambial activity can be

stimulated by mechanical pressure (Ko *et al*, 2004; Brown & Sax, 1962). However, little is known about physical stress patterns in the cambium, partly because the visualization of microtubule orientation is challenging in the thickening stem.

Modifications of cell wall properties can also be translated into morphogenic cues both in the SAM (Peaucelle *et al*, 2011; Yang *et al*, 2016; Sampathkumar *et al*, 2019) and at the cambium (Siedlecka *et al*, 2008). Sensing cell wall status might not only be a matter of mechanics but could also involve specific pathways capable of monitoring cell wall integrity through membrane localized receptors such as FER or THE (Wolf *et al*, 2012; Vaahtera *et al*, 2019). Mechanosensing ion channels are also capable of sensing membrane tension, and mechanosensing proteins have been identified in plants (Hamant & Haswell, 2017). However, it remains unclear to date how mechanical forces could be translated into a molecular or, more specifically, transcriptional response in the nucleus. Although many genes can be induced simply by touch (Xu *et al*, 2019; Van Aken *et al*, 2016; Fernie, 2019), so far the expression of only three genes has been correlated with the quantity of mechanical stimuli: *CUC3* and *STM* in the SAM of *Arabidopsis* (Landrein *et al*, 2015), and *PtaZFP2* in the hybrid tree *Populus tremula* × *alba* (Coutand *et al*, 2009).

6. Aims of the study

The aim of this thesis is to provide an integrative and dynamic perspective on radial growth. The work is based on an interdisciplinary approach that explores and characterizes the molecular and mechanical control of plant secondary development by focusing on the following three main goals:

- Study how physical forces affect radial growth.
- Reveal specific characteristics of all tissues involved in secondary growth.
- Analyze the transcriptional regulatory network of cambial development.

7. Materials and Methods

Materials and Methods and methods used in the publications included in this thesis. Methods performed by co-authors are indicated with brackets in the corresponding publications. Methods assisted by co-authors are indicated with an asterisk.

Method	**Publications**
RNA extraction	I, II
Illumina RNA sequencing	(I), (II), (III)
Desorption atmospheric pressure photoionization-mass spectrometry measurements	(II)
Quantitative analysis of triterpenes	(II)
Infrared spectroscopy	(II)
Monosaccharides and cellulose composition analysis of cell walls	I*, (II)
Suberin staining	II
DNA extraction	I, III
Whole genome Illumina sequencing	(I)
Cell wall polymer immunolocalization	I*
Microtome sections	I, II, III
Cryosections	I, II
Pyrolysis-gas chromatography and mass spectrometry	(I)
Atomic force microscopy (AFM)	I*
Tensile strength testing	I*

Flow cytometry for ploidy determination	(I)
Genome analysis, linkage mapping and QTL analysis	(I)
Automated anatomical quantification with LithographX	I, III
Stem mechanical manipulations	I
Fluorescence-activated cell sorting (FACS) and microarray expression data	(III)
Molecular cloning	(III)
GUS staining	(III)
RNA in situ hybridization	(III)
PCR and qRT–PCR	(III)

8. Results and Discussion

8.1 Trees respond to vertical mechanical stress

Various studies have explored the growth response of tree stems to variation in stem posture (Bastien *et al*, 2013; Gerttula *et al*, 2015; Niez *et al*, 2018; Coutand *et al*, 2008). These cases involved a strong gravitropic response, where the angle of the stem changed in respect to the gravity vector, and were always associated with tension wood formation. However, tension wood formation requires metabolic reconfigurations in order to produce cellulose-rich cells (Gorshkova *et al*, 2015), making this special type of wood common in tilted stems or branches but rather scarce in vertically growing trees. Therefore, we hypothesized that upright standing trees might also adjust their radial growth depending on their aerial weight in a tension wood independent manner.

To test this hypothesis, we designed an experiment where upright standing birch trees were exposed to four mechanical conditions: the first consisted of supported trees (control); in the second, we added extra weight to supported trees; in the third, we added extra weight but to a semi-free moving tree; and in the fourth treatment, we pulled the stem up from the top in order to partially cancel its own weight.

Our results indicate that manipulating stem weight vertically affects the rate of radial growth with variable intensity along the stem (Alonso-Serra *et al*, 2020). Interestingly, the fastest growing sections were always the higher ones, but subtle effects were identified among the treatments. At the base, we found that adding extra weight to the stem did not promote radial growth unless the trees were free to move (i.e., the third condition). When the trees were free to move, the lower half of the stem grew faster than in the control trees with no added weight or in the supported trees with extra weight. Importantly, none of the cross-

sections analyzed after the mechanical treatments showed tension wood formation. These results suggest that vertically standing tree stems are able to systemically adjust their rate of radial growth in response to changes in the vertical weight. We identified this mechanism as “vertical proprioception” (Figure 4).

The analysis of the loss-of-function birch mutant *elimäki* (*eki*) further enhanced our understanding of this process. *eki* trees are characterized by a collapsing phenotype after approximately three months of normal vertical growth. Eventually these trees fall because they are unable to support their weight due to a thinner and softer stem. However, *eki* trees were not always thinner; instead, the stem had a similar thickness compared to WT during the first two months of growth before collapsing. This suggests that as the *eki* stem develops its diameter growth is progressively compromised. Thus, we hypothesized that the radial growth defects in *eki* are partially due to the weight of its own biomass. Using the same mechanical treatments as for WT trees, we showed that *eki* trees were unable to respond to a weight stimulus with radial growth; in other words, they lack a vertical proprioceptive response at the base.

Additional characterization of the mutant phenotype revealed that xylem differentiation was delayed in *eki* compared to WT stems and that these differentiating tissues were mechanically softer. The analysis of cell sizes along the stem revealed that while xylem fiber cells in the apical internodes were expanded in *eki* compared to WT, the same cell type was compressed at the base of the stem, making it thinner. Further studies are required to explain how the xylem differentiation defect can impact the final wood quality. For example, some studies suggest that pectins may play a prominent role in the mechanical properties and intrusive growth of fibers (Siedlecka *et al*, 2008).

Although no candidate gene has been so far identified for the *eki* phenotype, a mitochondria-associated pathway that is involved in thigmomorphogenesis was

transcriptionally missregulated in *eki*, suggesting that the *ELIMÄKI* locus might be directly or indirectly associated with touch-induced stimulus.

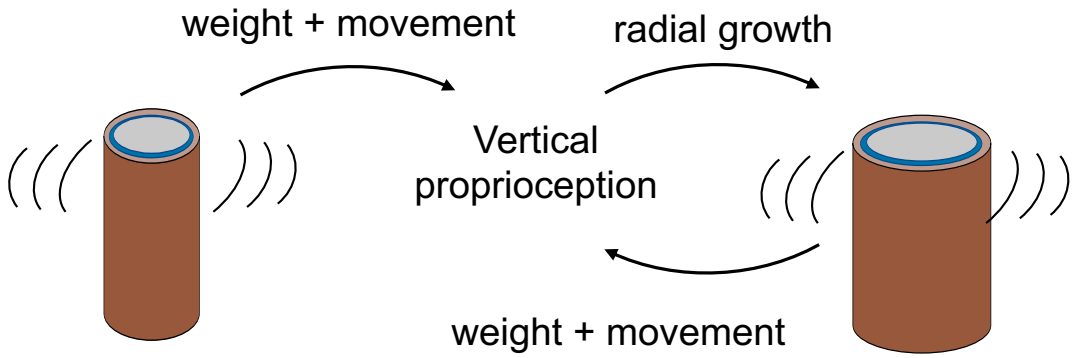

Figure 4: Vertical proprioception feedback loop. The combination of an increasing weight with lateral movement are transduced into a radial growth response. Growth also increases the weight, thereby feeding in the proprioceptive loop.

8.2 Molecular framework of bark development

Previous studies have studied the molecular networks expressed during wood formation in great detail and resolution (Sundell *et al*, 2017; Immanen *et al*, 2016). Similarly, multiple transcriptomic analyses have been performed in bark (Boher *et al*, 2018; Rains *et al*, 2018; Celedon *et al*, 2017; Mantello *et al*, 2014; Park *et al*, 2008). However, the lack of a holistic sampling approach to the stem can mask the level of specificity in the newly identified pathways. This is particularly important because the bark of trees consists of a unique array of tissues with different functions (Rosell *et al*, 2014). Unlike xylem, which is typically composed of three to four different cell types, bark tissues include cells dedicated to transport (sieve elements, phloem parenchyma and companion cells), mechanical support (phloem fibers) and defense against the environment (periderm cell types: phellem, phellogen and phelloderm). The specific

characteristics of each differentiated cell type are expected to be mirrored in their molecular profile: their transcriptome, cell wall and secondary metabolite composition. We therefore decided to explore the molecular variation across all the tissues, from the phellem to the mature xylem formed during the previous year of growth.

The first interesting result was that phellem had the highest number of tissue-specific transcripts, highlighting the fact that gene expression in this tissue is outstanding in the context of the stem. Up to 44 different yet interconnected biosynthetic pathways were identified in the phellem, including those involved in suberin and betulin production. While suberization of the phellem is a shared feature among many dioecious species ranging from *Arabidopsis* (Wunderling *et al*, 2018) to trees such as oak (Soler *et al*, 2007), poplar (Rains *et al*, 2018), alder and birch (Alonso-Serra *et al*, 2019), the production of betulin seems to have diversified mainly in the *Betula* genus. Although betulin is currently being investigated for various medical applications (Liby *et al*, 2007; Pisha *et al*, 1995; Sun *et al*, 1998), the function of this white-colored triterpene in the phellem remains unexplored. It has been hypothesized that it could reduce winter-induced damage in the bark by reflecting light and thereby reducing the internal stem temperature (Karels & Boonstra, 2003).

Another relevant finding was the shared molecular profile between the phellogen and the vascular cambium. Both lateral meristems were highly enriched in similar components of the cell cycle machinery, such as cyclins and cyclin-dependent kinases. While some TFs were expressed in both meristems, we also identified TFs specific to the phellogen such as *MYB66* (Lee & Schiefelbein, 1999) and *KANADI* (Kerstetter *et al*, 2001). These candidate genes could be used to further study whether the regulation of phellogen activity has an impact on cambial activity and to investigate whether these meristems are synchronized in terms of cell proliferation, or alternately, whether they conform to partially independent developmental modules (Tomescu & Groover, 2019).

8.3 Transcriptional regulation of cambial growth

Understanding the topography of gene regulatory networks provides a higher dimensional perspective on signaling pathways and could potentially guide the modification of cambial activity to enhance or restrict certain developmental processes. In order to identify cambium transcription factors, we performed cell sorting using root tissues and selected cells expressing the cytokinin-inducible procambium/cambium marker *pARR15*-GFP. Next, after *in silico* selection of 32 candidate TFs and validation of their expression profiles, we created a transcriptional regulatory network by testing their pairwise inducibility. To this end, individual inducible overexpression lines were developed, and gene induction was assessed by qPCR.

Our finding was that in contrast to other contexts, such as xylem differentiation, in which transcriptional regulation is known to be very hierarchical or pyramidal (Taylor-Teeples *et al*, 2015; Zhang *et al*, 2014), the transcriptional regulation of cambium activity appears to be highly redundant in terms of the TFs controlling the total cell number or diameter of the root (Zhang *et al*, 2019).

In this molecular network, four genes (*WOX4*, *PTL*, *KNAT1* and *LBD4*) were shown to represent highly interconnected nodes, and the loss of both *KNAT1* and *WOX4* was sufficient to reduce cambial activity to the minimum. However, residual cambium-like cells and xylem production were still observed in the double mutant.

We further implemented a high-throughput analysis of the loss-of-function phenotypes of the 32 TFs and their combinations, consisting of more than 100 genotypes. This approach demonstrated epistatic interactions among the TFs. When ranking the phenotype severity across genotypes, we found that several genotypes had a higher vessel number than the control (Col-0); however only one of them was slightly thicker (*erf072-1 myb87-2*). This contrast suggests that

radial growth and xylem differentiation can be genetically uncoupled and may not always correlate with each other. In fact, *WOX4* overexpression promoted cell proliferation, and this phenotype was further enhanced by the loss of *PTL*, but there was no effect on the number of differentiated xylem cells.

Taken together, these analyses suggest that in order to promote secondary growth towards enhanced wood formation, a candidate approach must take into consideration the balance between cell proliferation and differentiation. One example would be overexpressing a cell proliferating factor and knocking out a negative regulator of differentiation. Interestingly, whereas the gain or loss of no single TFs appears to reach such a goal, enhanced woody biomass production can be achieved when higher order (upstream) molecular regulation is manipulated in a tissue-specific manner in trees (Etchells *et al*, 2015; Immanen *et al*, 2016).

9. Conclusions and perspectives

Throughout this thesis, I have aimed to study different aspects that contribute to the robustness of plant secondary development.

In the first publication, our forward genetics approach with birches and the study of a loss-of-function phenotype led to the experimental demonstration of the developmental response called “vertical proprioception”. We were able to show that trees not only reinforce their base after a vertical mechanical stimulus but also display a systemic response that takes place along the stem, thereby reinforcing the entire organ. Furthermore, we showed that a vertical proprioceptive response is enforced through lateral stem movement.

Thus, when dissecting which factors contribute to the size of trees, we have added an obvious yet often unconsidered variable: their own weight.

Additionally, future studies of tree development will benefit from taking into consideration the experimental conditions in which trees are grown. For example, poplar trees are often grown supported, but such an experimental design may mask interesting secondary growth phenotypes. Additionally, measuring the diameter at multiple heights along the stem provides a more holistic model of secondary stem development than a single measurement.

The characterization of mutant *eki* trees further revealed how mechanical stress patterns are manifested in the stem. We found that the phenotype of cellular compression in *eki* had a cylindrical shape and was pronounced towards the base. This pattern may indirectly reveal the radial compression that woody tissues experience during secondary growth. However, this phenomenon should be studied further and integrated into a three-dimensional model of stem architecture. In the future, larger populations are required for narrowing down the mapping window to the gene level and identifying the *eki* mutation.

An encouraging result was the finding that accelerated flowering is effective in birch and represents the fastest forward genetics technique in trees so far. However, further optimization of this method is required given that flowering, fertilization and seed production success appear to vary between birch species and cultivars. Along these lines, another aspect to be studied is what makes birches so exceptional in this matter and which other species could be induced to flower precociously.

In the second publication, we highlighted the cellular diversity across the stem of silver birch (*Betula pendula*). Given that the meristematic activity of phellogen cells is poorly understood, this work serves as a source of candidate genes which regulate periderm development. In fact, at the moment a complete stem transcriptome is missing in *Arabidopsis*. This work also revealed the expression profile of the betulin biosynthesis pathway. Dissecting these pathways, along with other mechanisms of secondary metabolite production, is important in order

to understand tree adaptation and bark evolution in higher geographic latitudes, which are highly susceptible to climate change.

Finally, in the third publication we addressed the functional redundancy in the transcriptional regulation of cambial activity. This work showed that *WOX4* and *KNAT1* are major players in cambium development; however, the gain-of-function phenotype of *WOX4* was quantitatively and qualitatively stronger than that of *KNAT1*. Interestingly, while *WOX4* overexpression triggered cell division across the entire root vasculature, the double overexpression of *WOX4* with *KNAT1* or *ANAC015* seemed to attenuate the unorganized cell divisions and restrict them to the cambial zone. This may suggest that these TFs regulate each other either directly or indirectly, as proposed by our network analysis. Additionally, overexpression of more than one TF may better orchestrate the positive effect on the cambial transcriptional network. Furthermore, cambial regulation is known to be integrated by different feedback loops (Fischer *et al*, 2019); thus, more in depth studies are required to dissect these molecular pathways.

Taken together, these studies expand our view of plants by emphasizing the three-dimensional and highly dynamic nature of secondary development. Secondary development creates inspiring and fascinating organisms such as trees and at the same time sustains the ecosystems that depend on them. With good promotion of scientific outreach and sufficient engagement from both the public and the scientific world, research such as this can reveal the wonderful mechanisms taking place in the life of plants. By assimilating this knowledge, we might become better able to view plants as a part of our communities.

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